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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

Maertens et al. Atty. Ref.: 2551-109; Confirmation No. 3515

Appl. No. 09/899,303 TC/A.U. 1648

Filed: July 6, 2001 Examiner: Bao Qun Li

For: PURIFIED HEPATITIS C VIRUS ENVELOPE PROTEINS FOR DIAGNOSTIC AND THERAPEUTIC USE

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June 7, 2004

Commissioner for Patents
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Sir:

SUBMISSION

Supplemental to the Amendment filed April 14, 2004, attached is a copy of the de Martynoff reference referred to on page 13 of the remarks of the Amendment.

Respectfully submitted,

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VIRAL HEPATITIS AND LIVER DISEASE

Proceedings of IX Triennial International Symposium on Viral Hepatitis and Liver Disease Rome, Italy, 21-25 April 1996

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EDIZIONI MINERVA MEDICA
TURIN 1997

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ISBN 88-7711-293-X

Analysis of post-translational modifications of HCV structural proteins by using the vaccinia virus expression system

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Abstract

In order to identify the epitopes which induce an immune response against hepatitis C virus (HCV) infection, we expressed the full-length sequence and different deletion forms of each HCV structural protein -the nucleocapsid and the two envelope glycoproteins- in several mammalian cell lines using recombinant vaccinia viruses. All post-translational modifications of the single proteins and of the Core/E1 and E1/E2 products of the polyprotein constructs were similar to those of the complete structural polyprotein core/E1/E2. The signal sequence furnished by the hydrophobic domain of the preceding region enabled translocation of the envelope proteins into the endoplasmic reticulum (ER). The E1 and E2 polypeptide backbones were modified with five and eleven high-mannose motifs, respectively. A shorter amphiphilic form (19 kDa) was co-expressed with the full-length Core protein (22 kDa). Analyses involving inhibition of specific steps of the glycosylation pathway clearly indicated that the single E1 protein is transported from the ER to a pre-medial Golgi compartment. Indirect immunofluorescence and confocal microscopy studies, however, indicated that the majority of the envelope glycoproteins finally segregated into an ER-like compartment. The C-terminal region of both envelope proteins favoured both the segregation into the Tx-114 membrane phase as well as the intracellular retention, as only C-terminally truncated versions of E1 and E2 were partly secreted into the extracellular medium.

Introduction

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion non-A, non-B, hepatitis and represents a major public health problem worldwide. The enveloped virus contains a single-stranded RNA genome of about 9.4 kb which comprises a single large open reading frame.

This sequence encodes a polyprotein of about 3000 amino acids (aa) which is co- and post-translationally cleaved into mature proteins. The cleavages liberating the structural proteins (between Core/E1, E1/E2, E2/P7 and E2-P7/NS2) from the viral polyprotein probably result from cellular signal peptidase activity[1-3], while the cleavages between the non-structural proteins are mediated by the HCV NS2/3 metalloproteinase and the NS3 chymotrypsin-like protease[4-7].

Several studies have documented the appearance of antibodies against the structural proteins of HCV relatively early after the onset of hepatitis. In order to understand the pathogenesis of persistent infection and to develop effective vaccines, it is of vital importance to identify the critical epitopes that elicit neutralizing antibody responses.

In contrast to several studies which reported only the characterization of the complete structural region of HCV, we have demonstrated the possibility to express each of these proteins as single units presenting similar posttranslational characteristics as those of the polyprotein cleavage products. To this end, a series of constructs completely or partly covering each structural HCV region were expressed in several mammalian cell lines using recombinant vaccinia viruses. The nucleocapsid (Core) and the two envelope proteins (E1 and E2) were analyzed by several approaches and tested with HCV-infected patient sera. Both envelope proteins were further characterized for their N-linked carbohydrates, and the intracellular location of the E1 protein was mapped by means of transport inhibition and immunolocalization studies.

Material and methods

Cloning of the HCV structural sequence

HCV cDNA covering the complete 5' structural region (nucleotides -12 to +2427) was amplified from the serum of a patient with chronic hepatitis C by reverse transcription followed by polymerase chain reaction (RT-PCR), using oligonucleotide primers specific for HCV subtype 1b and containing either initiation or stop codons in frame with the viral sequence (Maertens *et al*, Unpublished data, 1990).

These amplified constructs were inserted directly into bacterial plasmid vectors and entirely sequenced by the dideoxynucleotide chain termination method. The HCV cDNA sequences were subsequently used for expression studies and reconstruction of longer cDNA clones (Fig. 1). Internal deletion of the first E1 hydrophobic domain (spanning from Leu-264 to Val-287) was performed by overlap extension mutagenesis[8] starting from the HCCI-10A sequence (Maertens *et al*, Unpublished data, 1990).

Generation of recombinant HCV-vaccinia viruses

The vaccinia recombinant vectors used in this study were based on a modified version of pATA18[9], with an additional insertion containing the dominant *Escherichia coli* gpt gene under the control of the vaccinia I3 intermediate promoter. The C-terminal ends of several truncated cDNA fragments were inserted in frame with stop codons in the three reading frames (vector pgsATA-18) or with a sequence encoding a six-histidine tail to facilitate rapid and efficient purification by means of Ni^{++} chelation (vector pMS-66).

Human 143B (TK-) or rabbit RK_{13} monolayers were infected

with wild-type WR vaccinia virus and subsequently transfected with plasmid DNA using the calcium phosphate coprecipitation method. After homologous recombination, HCV-vaccinia viruses were selected through two rounds of plaque purification in medium containing mycophenolic acid[10, 11]. Large-scale preparations of HCV-vaccinia viruses were amplified on RK_{13} cells, and titers of infectious progeny were determined by plaque assays.

Analysis of the recombinant HCV proteins

Confluent monolayers of cells infected with the recombinant HCV-vaccinia viruses (m.o.i. 5) were lysed in a buffer containing 1% Triton as described by Stunnenberg *et al.*[9]. Recombinant proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using either patient sera or specific mouse monoclonal antibodies (MAbs). After metabolic labeling in the presence of [^{35}S] methionine, recombinant proteins were specifically bound to anti-envelope MAbs (4°C, overnight, rotating wheel) and immunoprecipitated with Dynabeads M-280 sheep anti-mouse IgG (Dynal, Wirral, UK) before being analyzed by SDS-PAGE autoradiography or Western blot.

Endoglycosidase H (endo H), glycopeptidase F (PNGase F), the inhibitors of glycoprotein synthesis, and the different lectins conjugated with digoxigenin were obtained from Boehringer-Mannheim (Mannheim, Germany) and reactions were performed according to the manufacturer's instructions. The presence of a glycosylphosphatidylinositol (GPI) membrane anchor was analyzed by Western blot using a GPI anchor detection kit (Oxford GlycoSystems, Abingdon, UK).

Detergent phase separation was performed with an ice-cold PBS buffer containing 2% Triton X-114 as described previously[12]. Proteins contained in each phase or in crude lysates were separated by SDS-PAGE and identified by immunoblot analysis. The localization of HCV E1 antigens in transfected HeLa cells (12 h p.i.) was determined by immunofluorescence microscopy as described by Ash *et al.*[13]

Results and discussion

Construction of recombinant vaccinia viruses expressing full-length or truncated versions of HCV structural proteins

In order to better understand the processing, glycosylation, and antigenicity of the HCV structural proteins, we cloned a series cDNA sequences partly or completely covering each predicted region and expressed the recombinant proteins in different mam-

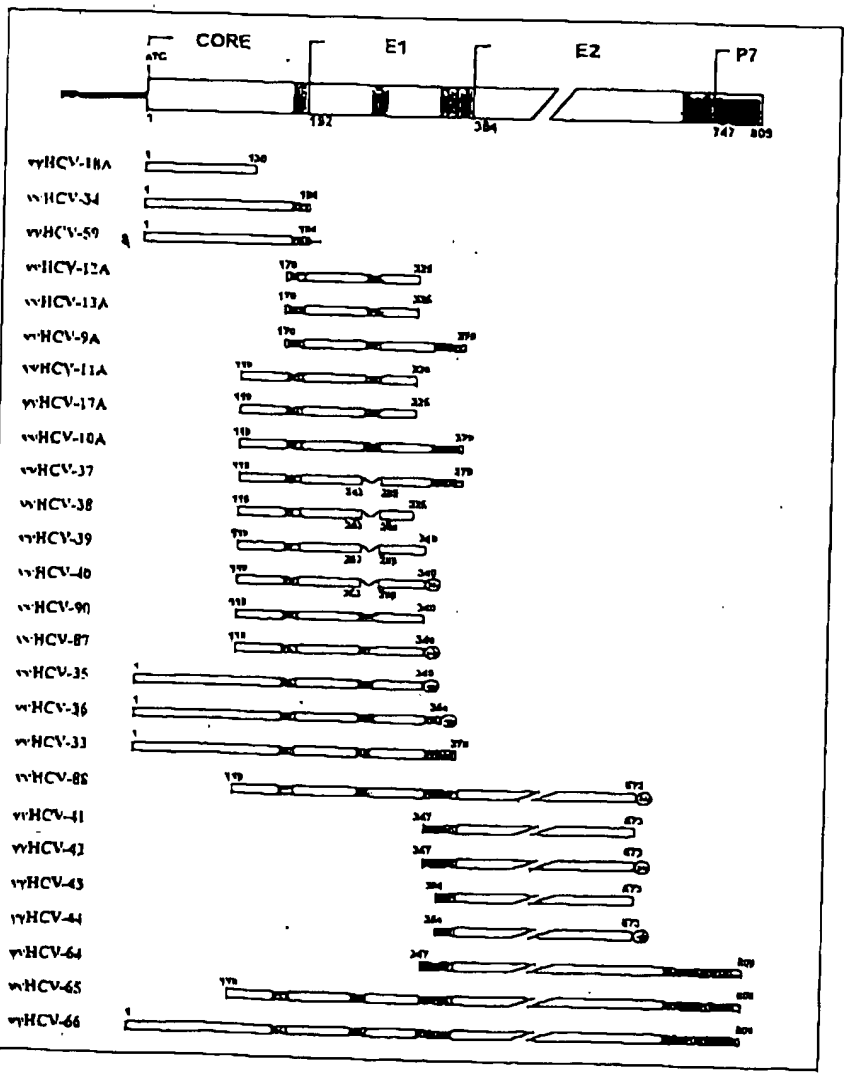


Fig. 1. — HCV vaccinia virus expression constructs from the HCV structural region. The organization of the structural region of the HCV polyprotein is depicted with the cleavage sites indicated by thick vertical bars. Numbers represent aa residues of the HCV polyprotein. Regions of the polyprotein containing predominantly uncharged amino acids/hydrophobic domains are indicated as shaded boxes. The designation and amino acid boundaries of the recombinant vaccinia viruses are indicated with respect to the polyprotein.

malian cell lines using the vaccinia virus system. To examine the processing of each protein independently from the possible influence of other viral proteins, single-protein constructs were compared with polyprotein constructs (C/E1, E1/E2, C/E1/E2, C/E1/E2/P7) (Fig. 1).

In correlation with other recombinant expression studies[1, 14], the single E1 and E2 cDNAs encoded a genuine HCV signal sequence provided by the carboxy-terminus of the preceding polyprotein region. Core and envelope deletion mutants were generated by PCR in order to remove signal-anchor sequences as well as other hydrophobic regions predicted to possibly interact with membranes (aa 131-191, aa 264-287, aa 327-383, aa 341-383, and aa 674-809) (Fig. 1).

When expressed in different mammalian cell lines (Fig. 2), signal endopeptidase processing and N-glycosylation of the single envelope proteins were similar to those of the structural polyproteins. In contrast with constructs encoding single envelope proteins (vvHCV-11A, -10A, -44; Fig. 2, lanes 4, 5, 6 and 8), expression levels decreased along with carboxyterminal position of E1 or E2 in polyprotein constructs (vvHCV-33, -65, -66; lanes 3, 8 and 9).

A shorter form is co-expressed with the full-length Core protein

From the majority of our HCV-vaccinia constructs analyzed, the Core protein was expressed in two forms, irrespective of the cell line (Fig. 3, lanes 2 and 4). One band migrated as expected for the full-length Core protein (p22), and another band was truncated to 19 kDa (p19). In contrast to other studies[15, 16], no 16-kDa protein (p16) was detected from our constructs in the absence or presence of the downstream E1 envelope sequence. The presence of the two Core forms does not seem to be due to either (i) a specific endoproteinase contaminant activated during cell lysis, (ii) binding to a RNA molecule, or (iii) the presence of two co-selected viruses each expressing one of the forms (data not shown). The second cleavage site generating p19 seems to be localized at the carboxyterminal region of the Core region, corresponding relatively well to the removal of approximately 18 residues, previously reported by Santolini *et al.*[17], and by Lo *et al.*[16]. However, only the p19 form could be detected in constructs encoding the entire structural region of the HCV genome (aa 1-809; vvHCV-66) (Fig. 2, lane 9). Therefore, the Core signal-anchor domain may require interaction with a downstream polyprotein region before further processing to a p19 form.

Using Triton X-114 phase separation, the two forms of Core segregated in both the detergent and water phases (Fig. 3, lanes 2 and 4). A shorter Core protein lacking its carboxy-terminal third (aa 1-130; vvHCV-18A) was unequivocally detected in the aqueous phase (artificial p16; Fig. 3, lane 3). Therefore, hydrophobicity of the p19 form was not drastically changed after removal of the short carboxy-terminus from the full-length p22.

An increasing number of amphiphilic proteins are reported to be anchored in the membrane bilayer by covalent attachment of the C-terminal amino acid residue to a glycosylphosphatidylinositol anchor. We therefore determined if this mechanism could be involved in the hydrophobicity of the Core p19 form. Lysates of cells infected with vvHCV-33 and -34 (Core/E1 and Core) were treated with a GPI-specific phospholipase C and analyzed by Western blot including the soluble or membrane forms of variant surface glycoprotein from *Trypanosoma brucei* as positive controls. When incubated in the presence of a polyclonal antiserum directed against a specific GPI-digested epitope (Oxford GlycoSystems, Abingdon, UK), no specific signal was detected for the recombinant Core proteins migrating at the expected 19- and 22-kDa molecular masses (data not shown). The results therefore suggested the absence of a GPI membrane-anchor modification on the Core p19 form.

Indirect immunofluorescence studies confirmed the localization of both core proteins exclusively in the cytoplasm, with a characteristic reticular/granular distribution[16-18].

Correct post-translational modifications of single and polyprotein envelope glycoproteins

Two series of recombinant E1 viruses were constructed containing either a short 22-aa or a longer 73-aa C-terminal domain of Core. In order to obtain an E1 protein secreted into the culture medium, truncated envelope cDNAs were generated by PCR to delete the short internal and/or the long carboxyterminal hydrophobic domains (Fig. 4A). Contrary to *in vitro* transcription translation studies in the presence of dog pancreas microsomes, the E1 constructs with the 73-aa signal sequence were markedly better expressed than proteins with a 22-aa signal sequence (Fig. 4A). Furthermore, the single E1 and E2 envelope proteins were produced

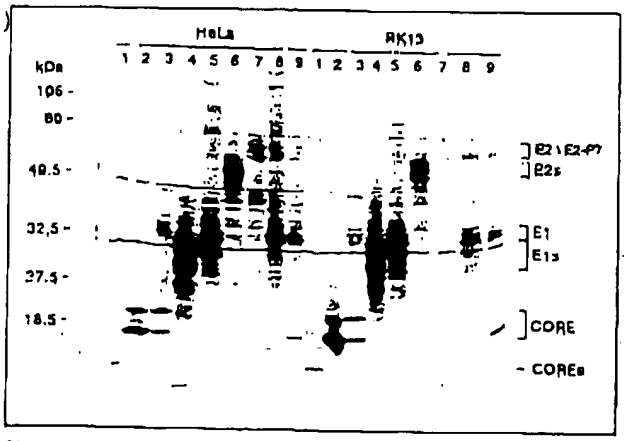


Fig. 2. — HCV structural proteins expressed by recombinant vaccinia viruses in two mammalian cell lines. Aliquots of infected HeLa or RK_{13} cells were separated on 13% SDS-PAGE and transferred onto nitrocellulose. Fillers were incubated either with 1/200 diluted serum of a HCV-infected patient (preincubated with 1/20 HeLa cell lysate), and with 1 mg/ml of supernatant from anti-E1 and anti-E2 MAbs. Lane 1, wt vaccinia virus; Lane 2, vvHCV-34; Lane 3, vvHCV-33; Lane 4, vvHCV-11A; Lane 5, vvHCV-10A; Lane 6, vvHCV-44; Lane 7, vvHCV-64; Lane 8, vvHCV-65; Lane 9, vvHCV-66.

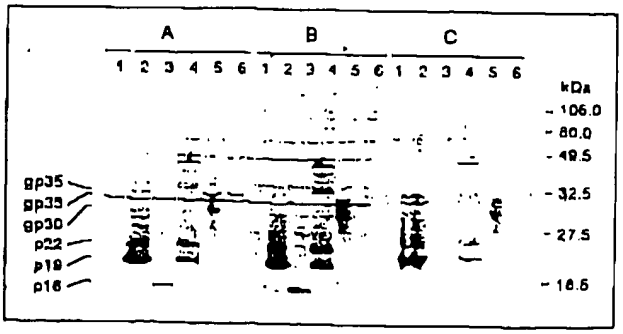
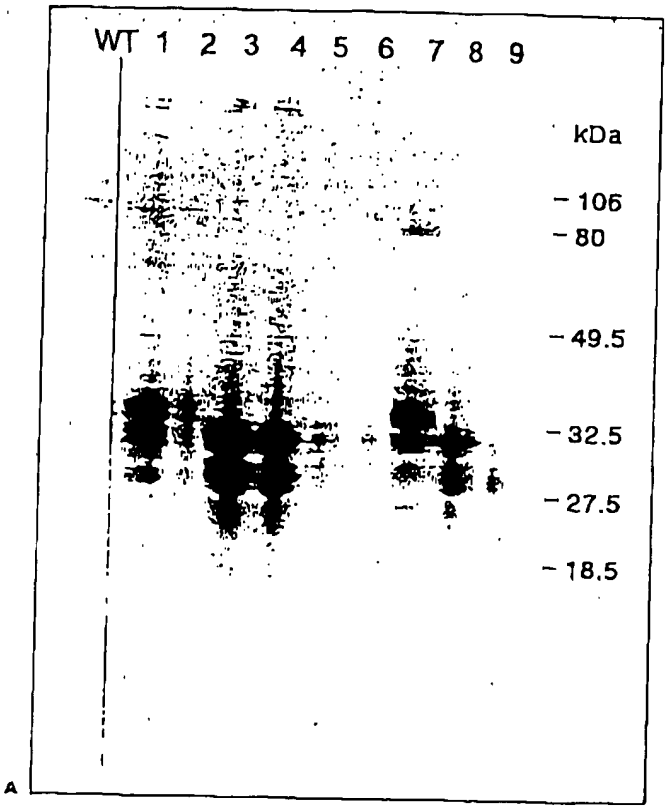


Fig. 3. — Triton X-114 phase separation of HCV structural proteins. 10^7 HeLa cells were infected and lysed in Triton X-114 buffer as described in Materials and Methods. Proteins from the crude lysate (A), or extracted in the aqueous (B) and the detergent phases (C), were separated on 13% SDS-PAGE and blotted onto nitrocellulose. Fillers were incubated with 1/200 diluted patient serum (preincubated with 1/20 HeLa cell lysate) and detected as described previously. Lane 1, wt vaccinia virus; Lane 2, vvHCV-34; Lane 3, vvHCV-18A; Lane 4, vvHCV-33; Lane 5, vvHCV-11A; Lane 6, vvHCV-10A.



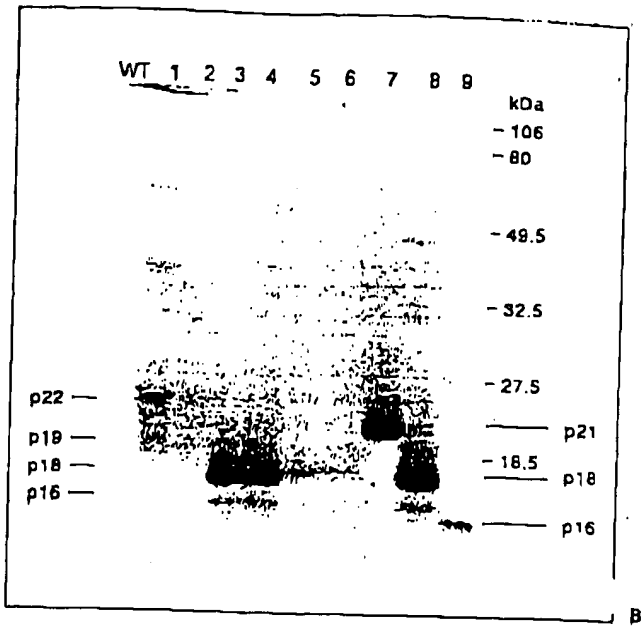


Fig. 4. — Analysis of HCV E1 constructs. Aliquots of infected HeLa cells (10^5 cells) were separated either directly on 13% SDS-PAGE (A), or after N-glycanase F treatment (0.5 mU PNGase F; 2 h at 37°C) on 15% SDS-PAGE (B). The filters were incubated with 1 mg/ml of an anti-E1 MAb and subsequently with 1/5000 diluted alkaline phosphatase-conjugated secondary anti-mouse antibodies. Lane WT: wt vaccinia virus; Lane 1, vvHCV-10A; Lane 2, vvHCV-33; Lane 3, vvHCV-11A; Lane 4, vvHCV-17A; Lane 5, vvHCV-12A; Lane 6, vvHCV-13A; Lane 7, vvHCV-37; Lane 8, vvHCV-39; Lane 9, vvHCV-38.

at higher levels as compared with the polyprotein constructs starting at the natural HCV initiation codon (vvHCV-33, -35, -36 and -66). A similar approach for the E2 protein revealed no difference between constructs containing either the 20- or 37-aa signal sequence (vvHCV-41 to -44).

Digestion of N-linked oligosaccharides with PNGase F indicated identical signal-peptidase cleavage sites in all E1 (at the core-E1 junction) and E2 proteins (at the E1-E2 border), irrespective of the length of the signal sequence (Fig. 4B, E_2 data not shown). Microsequencing of the amino-terminal residues of mature E1 and E2 polypeptides recovered from SDS-PAGE (data not shown) was in agreement with the deduced sequence from previous studies[1,2,6] and confirmed that Tyr-192 and His-384 are the amino-terminal residues of the processed E1 and E2 proteins, respectively.

The HCV E1 protein is transported to a pre-medial Golgi compartment

Endo H treatment and lectin binding studies confirmed the presence of high-mannose type glycans on the majority of potential N-glycosylation sites of the E1 molecules (Fig. 5A). Addition of N-glycan chains was clearly detected for the 5 sites of the carboxy-truncated E1 versions (the sixth putative site was deleted in this construct), but also for 5 [on 6 theoretically predicted sites in the HCV subtype 1b isolate] of the full-length E1 forms. By contrast, all the HCV E2 constructs (C-terminally truncated or containing the additional P7 sequence) bore N-linked sugar chains on each of their 11 potential glycosylation sites (Fig. 5B).

Western blot or pulse-chase experiments with a series of glycosylation inhibitors (Fig. 6) confirmed the addition of N-linked oligosaccharide precursors on the polypeptide backbone (tunicamycin). In the presence of castanospermine (inhibiting the ER-resident mammalian glucosidase I and blocking the glycoproteins in a higher apparent MW form) or 1-deoxymannojirimicin (inhibitor of the *cis*-Golgi α-mannosidase I), the electrophoretic mobility of this E1 glycosylated form shifted to higher apparent MWs of 40 and 37 kDa, respectively, by comparison with the 35-kDa protein expressed under normal conditions or in the presence of swainsonin (inhibitor of the medial-Golgi α-mannosidase II).

In contrast with many studies suggesting the retention of E1 in the ER compartment[2,3], the glycosidase activity of the *cis*-Golgi α-mannosidase I on E1 proteins clearly indicated a transport of the envelope protein from the ER to a pre-medial Golgi compartment. However, indirect immunofluorescence and confocal microscopy studies on HeLa cells expressing different forms of E1 showed further segregation into an ER-like compartment (data not shown).

In conclusion, the HCV E1 and E2 single envelope proteins exhibit properties similar to the native E1 and E2 proteins or E1E2 derived from polyprotein constructs. Because of their high yield expression, the single E1 and E2 glycoproteins may serve as ideal candidates for the development of new vaccines, diagnostics, and antivirals.

Acknowledgements

We are grateful to Jos Raymackers, Ann Wyseur, and Annemie Ducatteeuw for their excellent technical assistance.

Grant support

This work was supported in part by the Flemish Government, Ministry of Economics (Grants FIOV/IWT/91/007.tv] and ADV/94072/Innogenetics) as part of Eureka Project EU680 'hepatitis C'.

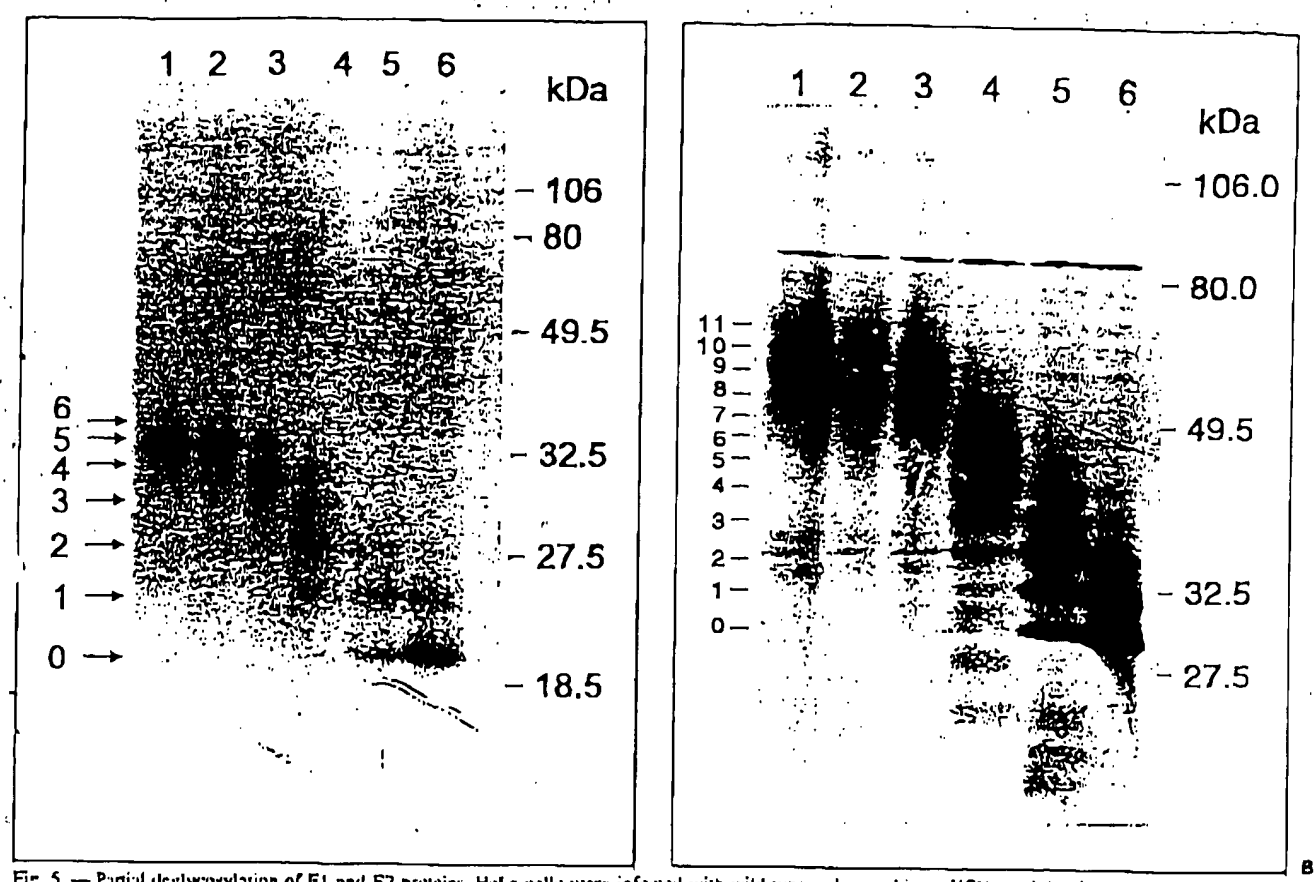


Fig. 5. — Partial deglycosylation of E1 and E2 proteins. HeLa cells were infected with wild type and recombinant HCV-vaccinia viruses vvHCV-10A (A) or vvHCV-44 (B). Aliquots of infected cell lysates (representing 10^6 cells) were digested for 2 h at 37°C with serial 10-fold dilutions of PNGase F (lane 6, 1 mU, to lane 2, 0,01 mU). Numbers at the left of the blot represent the number of N-glycan motifs added on the envelope polypeptide backbone.

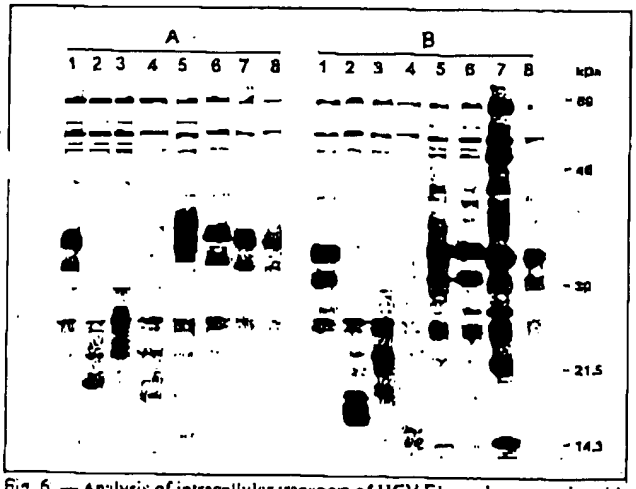


Fig. 6. — Analysis of intracellular transport of HCV E1 envelope protein with inhibitors of the N-glycosylation pathway. Confluent HeLa cells (10^6 cells) were infected with wild type (wt) or recombinant HCV E1-vaccinia viruses vvHCV-10A (A) or vvHCV-11A (B) overnight before being incubated for 3 h in the presence of N-glycosylation inhibitors. Cells were lysed and E1 was immunoprecipitated overnight with anti-E1 MAbs and anti-IgG coupled to magnetic beads. Lane 1, untreated; Lane 2, digested with 2 mU Endo H; Lane 3, treated with 0.5 mU PNGase F; Lane 4, 10 mg/ml tunicamycin; Lane 5, 20 mg/ml castanospermine; Lane 6, 200 mg/ml 1-deoxymannojirimycin; Lane 7, 5 mg/ml swainsonine; Lane 8, 10 mg/ml brefeldin A. The cells were starved in methionine-free DMEM for 1 h at 37°C before being pulsed with 100 mCi of ^{35}S-methionine (Amersham) for 30 min, washed twice, and chased with an excess of cold methionine for 5 h (all steps in the presence of glycosylation inhibitors).

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